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Improved methods of measuring the latent HIV reservoir with DNA size selection and droplet digital PCR

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Thesis

IMPROVED METHODS OF MEASURING THE LATENT HIV RESERVOIR WITH DNA SIZE SELECTION AND DROPLET DIGITAL PCR

by

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DEDICATION

I would like to dedicate this work to Kobe and the entire Bryant family.

ACKNOWLEDGMENTS

Nothing but genuine appreciation for my family, friends, and mentors for guiding me and helping to make all of this possible.

IMPROVED METHODS OF MEASURING THE LATENT HIV RESERVOIR WITH DNA SIZE SELECTION AND DROPLET DIGITAL PCR

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ABSTRACT

For HIV-infected patients undergoing antiretroviral treatment (ART), the latent reservoir is a major barrier to cure. Formed in the acute stages of infection, the latent reservoir consists of cells in the host's body where HIV can hide from the immune system and remain in a quiescent state. When a patient stops ART, the virus quickly rebounds, proliferates, and begins infecting more cells. The majority of assays available to measure the latent HIV reservoir measure HIV DNA and RNA using polymerase chain reaction (PCR). However, these methods can overestimate the reservoir size, because unintegrated and replication-incompetent DNA comprise a larger proportion of HIV nucleic acids than does replication competent HIV provirus.

To measure integrated HIV DNA from infected donor samples provided by the HIV Reservoir Assay Validation and Evaluation Network (RAVEN) and The University of Texas Health Sciences Center at Houston (UTHealth), I applied pulsed-field gel electrophoresis (PFGE) to reduce the amount of unintegrated HIV DNA such as episomal 2-long-terminal-repeat (2-LTR) circles before

quantitation with droplet digital PCR (ddPCR). This assay could prove useful to measure changes in the latent HIV reservoir, especially in clinical trials that aim to reduce its size through a variety of compounds.

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LIST OF ABBREVIATIONS

1-LTR.....1-Long Terminal Repeat

2-LTR.....2-Long Terminal Repeat

AAV ..... Adeno-Associated Virus

AC..... Alternating Current

AIDS..... Acquired Immunodeficiency Syndrome

ART ..... Antiretroviral Therapy

CNS ..... Central Nervous System

CRISPR ..... Clustered Regularly Interspaced Short Palindromic Repeats

CTL..... Cytotoxic T Lymphocytes

DC.....Direct Current

dCA ..... Didehydro-Cortistatin

ddPCR..... Droplet Digital Polymerase Chain Reaction

DI ..... Deionized

DMSO ..... Dimethyl Sulfoxide

DNA ..... Deoxyribonucleic Acid

ELISA ..... Enzyme-Linked Immunosorbent Assay

FBS..... Fetal Bovine Serum

GI ..... Gastrointestinal

HDAC ..... Histone Deacetylase

HIV ..... Human Immunodeficiency Virus

iCARED ..... Inducible Cell-Associated RNA Expression in Dilution

IUPM ..... Infectious Units Per Million

KAP1 ..... KRAB-Associated Protein 1

KRAB ..... Kruppel-Associated Box

MAC ..... Mycobacterium Avium Complex

NF- κB ..... Nuclear Factor- κB

NFAT ..... Nuclear Factor of Activated T Cells

NK ..... Natural Killer

OIs ..... Opportunistic Infections

PBS ..... Phosphate-Buffered Saline

PFGE ..... Pulsed Field Gel Electrophoresis

PHA ..... Phytohemagglutinin

PKC ..... Protein Kinase C

PMA ..... Phorbol Myristate Acetate

QVOA ..... Quantitative Viral Outgrowth Assay

RAVEN ..... HIV Reservoir Assay Validation and Evaluation Network

RNA ..... Ribonucleic Acid

sgRNAs..... Single-Guide RNAs

TB.....Tuberculosis

INTRODUCTION

Background

Before the introduction of ART in the late 1980s, infected individuals with HIV rarely survived for more than ten years. ART refers to any HIV treatment regimen that uses a combination of two or more drugs to combat the infection. Patients on ART with different combinations of protease, reverse transcriptase, and integrase inhibitors can have a close to normal life span and quality of life. However, ART can cause adverse long-term side effects, which can be problematic in decreasing patient adherence to the drug regimens. Figure 1 illustrates key steps in the HIV replication cycle cascade targeted by inhibitors to halt the production of viral particles. These steps include envelope fusion, RNA reverse transcription, and integration into the host genome (Arts & Hazuda, 2012).

HIV morbidity has been decreased dramatically due to ART, but a significant number of people with limited or no access to treatment still die each year. In addition, there was an estimated 1.7 million new infections in 2019 alone, according to UNAIDS. Without medication, HIV rapidly destroys the immune system and progressively evolves to Acquired Immunodeficiency Syndrome

(AIDS). Those who have arrived at this end of stage of HIV infection become extremely prone to opportunistic infections (OIs) by bacteria, viruses, fungi, and parasites. Pneumococcal pneumonia, tuberculosis (TB), and Mycobacterium avium complex (MAC) infections are just a few examples of deadly illnesses that can arise as a result of the body's weakened immune system (Benito et al., 2012). The body's compromised immune surveillance also makes it more difficult to clear these opportunistic infections than an otherwise immunocompetent individual.

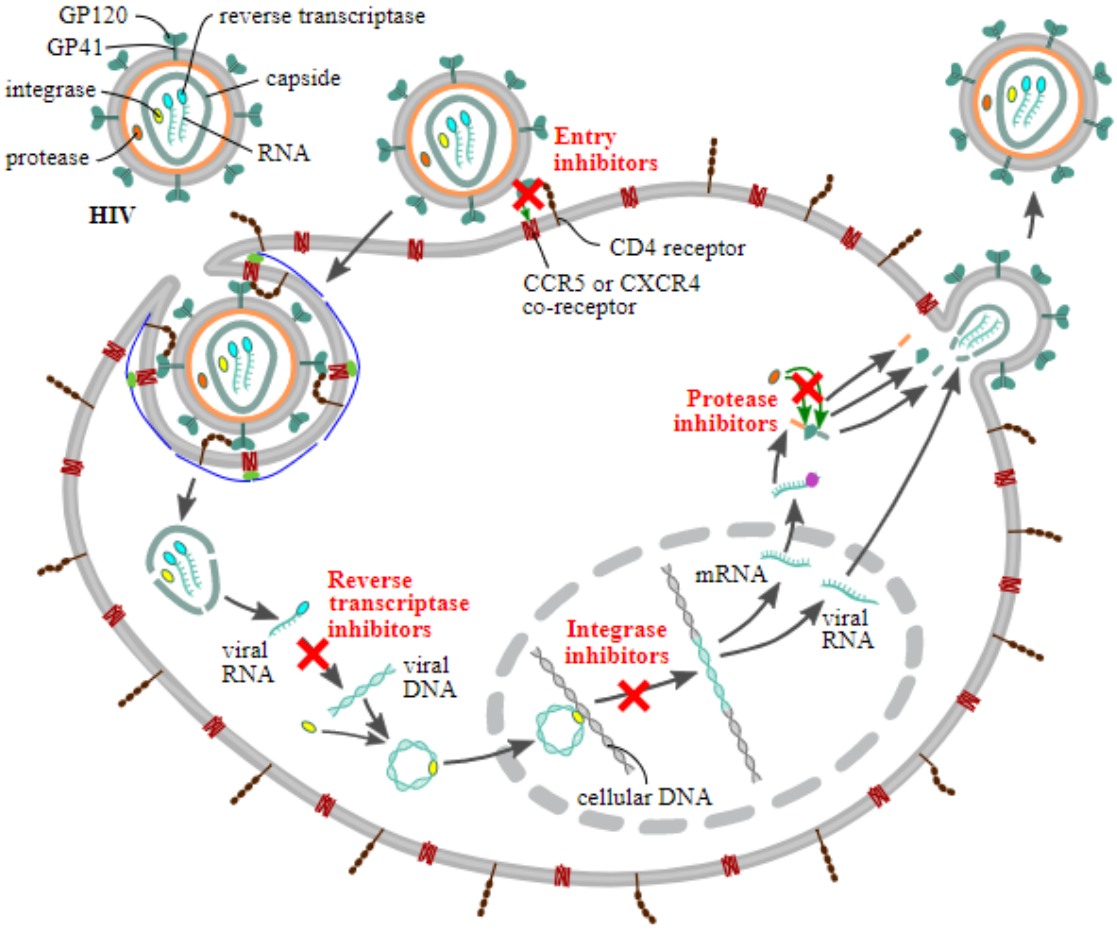

Figure 1. Schematic Description of the Mechanism of the Four Classes of Currently Available Antiretroviral Drugs against HIV.

Fusion inhibitors (interfere with the binding, fusion or entry of an HIV virion), reverse-transcriptase inhibitors (interfere with the transcription of viral RNA into DNA), integrase inhibitors (block the viral enzyme integrase, that inserts the viral genome into the DNA of the host cell), protease inhibitors (block proteolytic

cleavage of protein precursors that are necessary for the production of infectious viral particles). Taken from (Splettstoesser, 2013).

HIV Latency

ART prevents ongoing HIV replication, but once it is disrupted, HIV rapidly rebounds and begins to infect other cells again. This suggests the existence of a latent reservoir – a sanctuary site in previously infected cells for dormant viruses to hide in. By definition, a latent viral reservoir can preserve some quantity of replication competent virus to provide the capacity to continue future progenies (Saksena et al., 2010). Furthermore, the latent HIV reservoir is established quite early during the acute phase of infection and persists even when the viral load is undetectable in blood (Vanhamel et al., 2019).

Many clinical studies have been conducted to assess the natural decay of the latent HIV reservoir. In one cohort of 30 to 101 individuals, the reservoir half-life was estimated to be around 44 months. In another cohort of 101 individuals on ART and virally suppressed for a minimum of 4 years, the half-life was estimated to be closer to 13 years. This suggests that without a cure, the "natural" eradication of HIV from the body seems highly unlikely in one's lifetime (Bachmann et al., 2019).

The latent HIV reservoir exists both cellularly and anatomically. Figure 2 illustrates several key sites of the latent HIV reservoir, but it is still unclear whether all of these sites contribute to viral rebound once ART has stopped. Cellularly, it is widely agreed upon that the resting memory CD4+ T lymphocyte population is the major site for HIV persistence. On average, it is estimated that one in one million CD4+ T lymphocytes is latently infected with replication-competent virus (Hodel et al., 2016). As the primary target of HIV infection, this is also the reason why there is rapid destruction and depletion of CD4+ T lymphocytes in afflicted individuals (Saksena et al., 2010). HIV enters the cell primarily through CCRR5 or CXCR4 co-receptors and then CD4 receptors on the CD4+ T lymphocyte surface. Binding to these leads to subsequent fusion of the viral and host cell membranes and entry of viral proteins and nucleic acids.

Anatomically, HIV spreads through the bloodstream to different areas of the body within a couple of days. Infected cells have been discovered in the lungs, kidneys, gastrointestinal (GI) tract, reproductive tract, as well as the central nervous system (CNS). While some anatomical sites are relatively easy to reach with current drug regimens, the CNS is relatively protected by the blood-brain barrier, which creates challenges for ART and cure strategies.

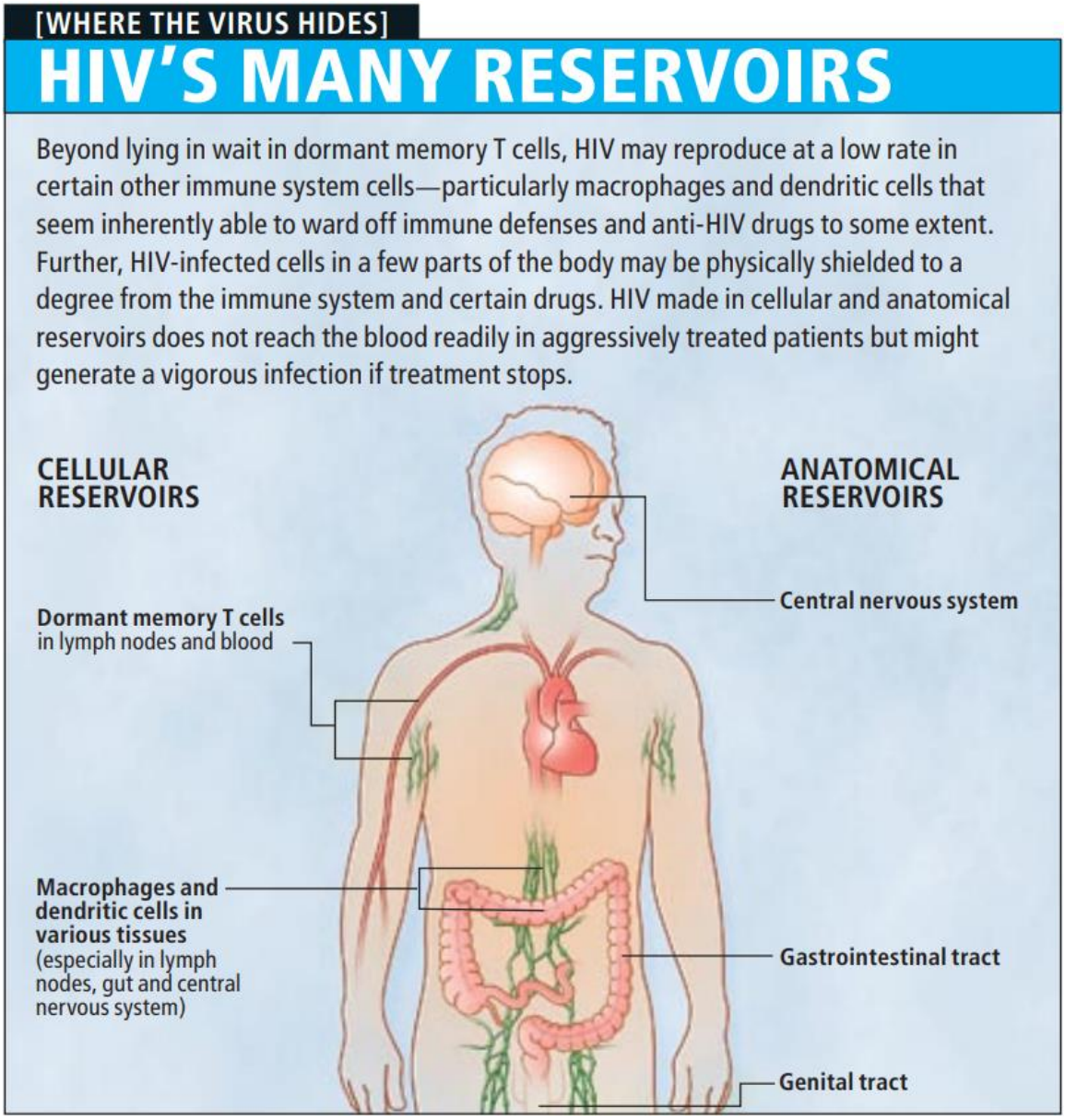

Figure 2. Reservoirs of HIV-1 Persistence in HAART.

Taken from (Stevenson, 2008).

Once the latent HIV reservoir has been established, it can be maintained by several mechanisms illustrated in Figure 3. For example, transcriptional factors required in the nucleus such as Nuclear Factor- κB (NF- κB) or Nuclear Factor of Activated T Cells (NFAT) can be sequestered in the cytoplasm, leading to transcriptional silencing. Furthermore, epigenetic regulation by Histone Deacetylases (HDACs) and Histone Methyltransferases (HMTs) can reinforce a strong transcriptional block. Finally, transcription elongation factors such as the Transcriptional Elongation Factor B (p-TEFB) Complex can also be sequestered such that they become catalytically inactive.

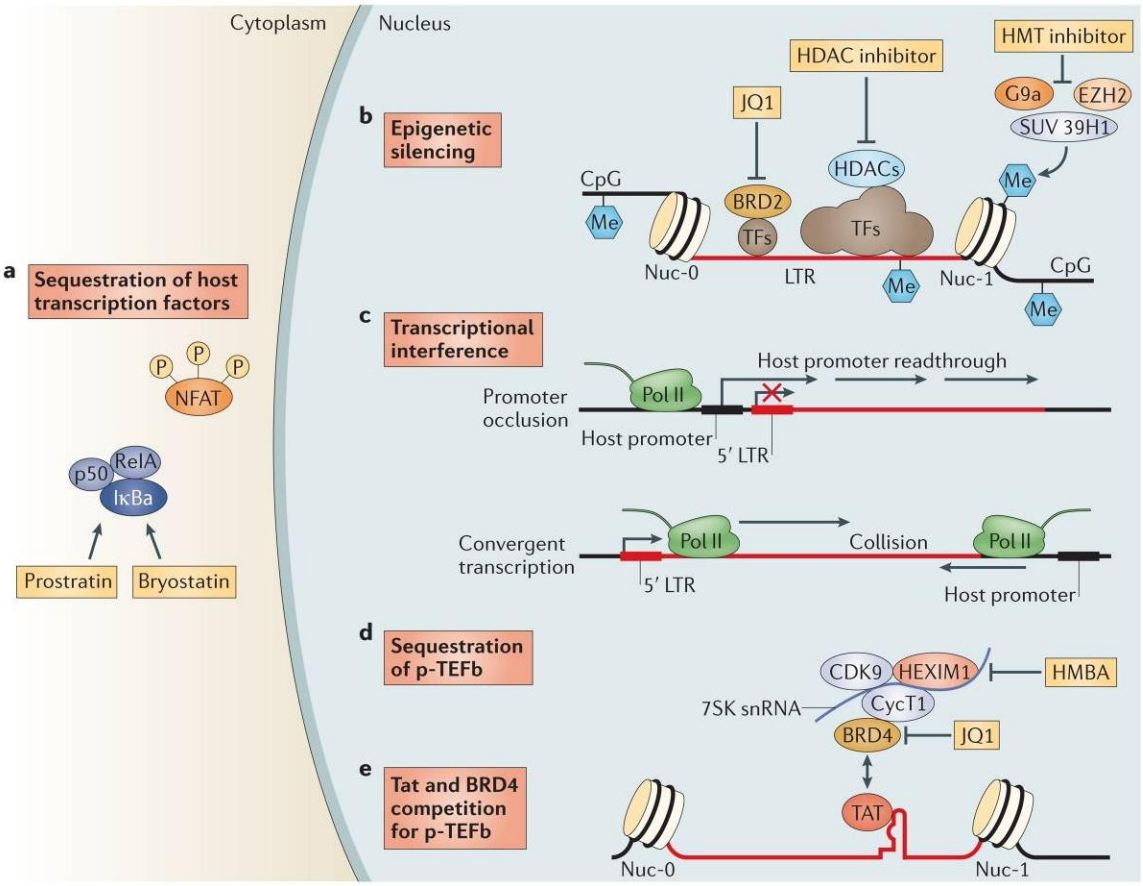

Figure 3. Mechanisms Involved in the Maintenance of HIV-1 Latency and Strategies to Disrupt Latency.

Taken from (Archin et al., 2014).

Cure Strategies

Currently, there are approaches to HIV cures in development: a sterilizing cure and a functional cure. The former aims to completely remove HIV from the body whereas the latter aims to enable the body to control viral replication on its own. However, the existence of a latent reservoir has become a major barrier to cure efforts and many strategies have shifted focus in an attempt to reduce its size.

One method proposed to destroy the latent HIV reservoir is the "Shock and Kill" or "Kick and Kill" strategy. This approach combines latency reversal agents (LRAs) to activate latently infected cells while continuing ART to prevent new infections. The reawakened cells would once again begin viral transcription and translation, and allow cytotoxic CD8+ T lymphocytes (CTLs) or natural killer (NK) cells to identify them and flush them out of the body. Notable LRAs include protein kinase C (PKC) agonists and histone deacetylase (HDAC) inhibitors, which upregulate HIV transcription through signal cascades or epigenetic modification of chromatin, respectively (Castro-Gonzalez et al., 2018). in preliminary trials with HIV-infected humanized mouse models on ART suppression, it was shown that the 3B3-PE38, an immunotoxin that targets HIV envelope glycoproteins, can be used to deplete latently infected cells in vivo

(Denton et al., 2014). Of course, limitations to this approach cannot be neglected. LRAs can be inefficient at disrupting latency in certain areas of the body and side effects of these compounds have not been studied extensively.

Recently, CRISPR/Cas9 has been proposed for an HIV cure as gene therapy garners more and more attention in the biomedical field. The CRISPR/Cas9 complex – originally observed in bacteria as a defense against viruses – could bind to single-guide RNAs (sgRNAs) transcribed from palindrome sequences of the host genome and make endonucleolytic cuts in complementary DNA sequences. DNA repair mechanisms are activated, and the double-stranded DNA breaks can be modified by deletion or insertion, then integration. Researchers have successfully excised integrated HIV provirus in animal models using an adeno-associated virus (AAV) vector to deliver single-guide RNAs and the Cas9 endonuclease derived from Staphylococcus aureus (Yin et al., 2017). Results demonstrated significant deletions of HIV proviral DNA in a number of tissues – including the brain, lungs, kidneys, and spleen.

Since then, there has been additional research conducted to repurpose the Cas9 endonuclease for an "Lock and Block" strategy. Illustrated in Figure 4, a catalytically inactive Cas9 endonuclease is paired with repressors targeted at specific HIV promotors to modulate its transcriptional activity. Specifically,

Kruppel-associated box (KRAB) zinc-finger proteins have been reported to repress HIV transcription through further signaling cascades featuring KRAB-associated protein 1 (KAP1) and other corepressors (Olson et al., 2019). With the ethical issues surrounding gene editing, this repurposed approach to Cas9 may offer a more reasonable and acceptable method of epigenetically repressing the production of new virions.

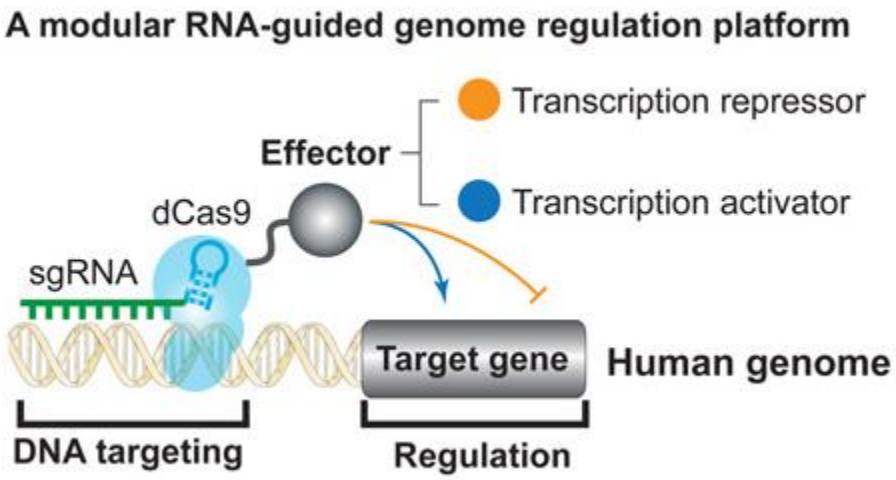

Figure 4. A Modular RNA-Guided Genome Regulation Platform.

dCas9 fused to effector domains can serve as an RNA guided DNA binding protein to target any protein to any DNA sequence. Taken from (Gilbert et al., 2013).

Similarly, other compounds such as Didehydro-Cortistatin (dCA) have also been explored in HIV-infected humanized mouse models for the delay and reduction of viral rebound after ART interruption. Compared to viral suppression under ART, dCA was shown to promote further epigenetic silencing by inhibiting the Tat protein from recruiting necessary transcriptional factors to the HIV promoter (Kessing et al., 2017).

Measuring Changes in the Latent HIV Reservoir

In order to validate the effectiveness of existing and future cure strategies, it is necessary to have assays that can measure changes to the HIV latent reservoir with accuracy, precision, and reproducibility.

The gold standard for quantifying the size of the latent reservoir is the quantitative viral outgrowth assay (QVOA). Illustrated in Figure 5, the QVOA is a terminal dilution culture assay in which resting CD4+ T lymphocytes are reactivated with an LRA. The virus is then expanded using uninfected cells and the amount of viral protein p24 is assessed with an enzyme-linked immunosorbent assay (ELISA) (Massanella & Richman, 2016). Ultimately, the results can be reported as an Infectious Units per Million (IUPM) value with a 95% confidence interval (Rosenbloom et al., 2015). Compounds used to reverse

latency have included phytohemagglutinin (PHA), antibodies for CD3/CD28, and Phorbol Myristate Acetate (PMA) paired with Ionomycin. Uninfected cells for viral amplification can range from HIV-negative CD4+ T lymphocytes and human T lymphoblast cell lines such as MOLT-4.

However, besides being time-consuming, labor-intensive, and expensive, the QVOA significantly underestimates the size of the latent HIV reservoir. This underestimation largely stems from the incomplete activation of latently infected cells in culture; it has been reported that nearly 12% of proviruses that are replication competent, are not successfully activated by LRAs like PHA (Ho et al., 2013).

The latent HIV reservoir may also differ between different cellular or anatomical sites. The frequency of HIV genomes was shown to be higher in transitional and central memory cells than effector memory cells (Murray et al., 2016). Both the ileum and rectum had higher HIV DNA levels in CD4+ T lymphocytes than blood (Yukl et al., 2013). Patient-to-patient variability also contribute greatly to the variable efficacy of LRAs.

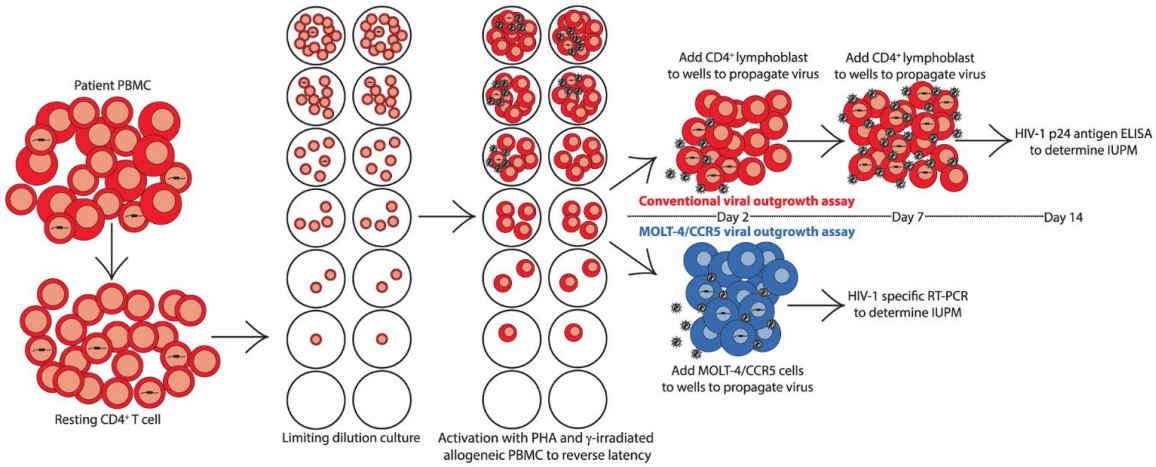

Figure 5. The Standard and Modified QVOA.

Taken from (Laird et al., 2013).

PCR assays are faster, cheaper, and require less cells than QVOA because they analyze more frequent events. However, the detection of total HIV DNA by PCR overestimates the size of the latent reservoir, because PCR assays also detect forms of unintegrated DNA such as 1-LTR, 2-LTR circles, and also replication incompetent proviruses.

To account for these unintegrated DNA fragments, the Alu-gag qPCR was introduced. This has been the most common method of measuring truly integrated HIV DNA in the latent reservoir. In the first step, one primer is anchored to Alu repeat elements in the human genome while the other is anchored to gag, an HIV gene that encodes to the structural components of the

virion. In the second step, primers within the HIV long terminal repeat (LTR) region will detect HIV specific products that can be quantitated by comparing the resultant signals to those from a standard (Liszewski et al., 2009). Thus, while unintegrated HIV DNA is still linearly amplified, integrated HIV DNA is preferentially and exponentially amplified, leading to much stronger signals.

In contrast to the traditional qPCR, ddPCR utilizes a water-oil emulsion droplet system which allows for DNA templates to be individually partitioned. PCR amplification is then carried out within each droplet, leading to increased precision, higher signal-to-noise ratios, and the removal of PCR bias.

RNA assays, such as the Inducible Cell-Associated RNA Expression in Dilution (iCARED) assay, are also promising methods of measuring the latent HIV reservoir. This method more sensitive, faster, and has a higher throughput than the standard QVOA. Similar to the QVOA, CD4+ T lymphocytes are isolated and plated in three-fold dilutions in the presence of Raltegravir to prevent viral propagation. After a three-day culture, both cell-associated and cell-free RNA are extracted with an automated platform and magnetic beads. Then, ddPCR is used to target specific sequences such as gag or tat/rev and an IUPM value is calculated. The regions of tat/rev are only present in HIV fragments that have completed post-transcriptional splicing and indicate more

likely replication competence. Compared to the QVOA, the iCARED assay reports a latent HIV reservoir size 13 times larger. Compared to the total HIV DNA assay, the iCARED assay reports a latent HIV reservoir size 19 times smaller. (Massanella et al., 2015).

Table 1 illustrates a summary of the aforementioned assays to measure the latent HIV reservoir. When time is a factor, PCR assays are generally more preferable than culture assays. When higher sensitivity is required, ddPCR should be the detection method of choice. Each method has its pros and cons, but it is crucial to the development of an HIV cure that these assays have the ability to accurately and precisely detect changes in the latent reservoir

	Assay	Detection method	Days	Pros	Cons
PCR assays					
HIV DNA	Total HIV DNA	qPCR or ddPCR	1	Easy, fast, and sensitive. Does not require cell culture.	The majority of proviruses detected are defective.
	Integrated proviral DNA	qPCR		Direct cell lysis reduces input cell numbers. ddPCR more precise than qPCR and absolute quantification.	
	2-LTR circles, as a marker of residual replication	qPCR or ddPCR		Total HIV DNA; quantifies integrated and unintegrated HIV DNA (i.e., 2-LTR Circles)	
HIV RNA	us	qRT-PCR or ddPCR	1	Easy	Does not quantify all latently infected cells.
	ms	qRT-PCR or ddPCR		Does not require cell culture	Less sensitive than HIV DNA
	Poly-A	qRT-PCR or ddPCR		Poly-A: detects fully elongated HIV transcripts	Detection of defective proviruses
Residual viremia		qRT-PCR	1	Measure of residual virus production during ART	Time consuming; Low dynamic range; Close to the limit of detection (with 8 ml of plasma); Does not directly measures the latent reservoir
Culture Assays					
QVOA	Replication competent virus after activation and propagation of the infection with pooled CD8-depleted PBMCs from uninfected donors or the MOLT.4/CCR5 cell line	p24 antigen ELISA or RT-PCR in supernatant to measure cfRNA in supernatant	9–21	Measures only replication-competent virus; Increased sensitivity for RT-PCR assay compared with the p24 detection, without detection of defective proviruses; The use of MOLT-4/CCR5 reduces time of culture	Long protocol; Low dynamic range; Great variability due to donor variability (improved with MOLT-4/CCR5); Requires large number of cells; Not all intact proviruses get reactivated
Inducible transcription assays	Proviruses that can be induced after activation to make cell-associated RNA (us or ms)	RT-PCR or ddPCR for caRNA (us or ms)	2–7	No need for outgrowth of virus before measurement; Faster than QVOA; ddPCR more precise than RT-PCR; TILDA: requires less cells	May detect some defective proviruses. Does not measure replication competent viruses.
	Proviruses that can be induced after activation to make cfRNA	RT-PCR or ddPCR for cfRNA	2–7	No need for outgrowth of virus before measurement; Faster than QVOA; ddPCR more precise than RT-PCR; Confirms antigen production as well as transcription; Measures transcription and translation, and most cell free virions are replication competent	May detect some defective viral particles. Proportion of QVOA + cell cells produce cfRNA.

Table 1. Characteristics of Assays for Measuring the Latent HIV Reservoir.

Taken from (Massanella & Richman, 2016).

SPECIFIC AIMS

Although the Alu-gag qPCR assay is widely used for measuring integrated viral DNA, there are certain limitations to this method. First, this assay requires a large quantity of DNA material for downstream amplification. Second, there is uncertainty as to the distance between the Alu sequence and the site of HIV integration and many believe that this could lead to the underestimation of integrated gag copies. Finally, qPCR has a lower sensitivity for the detection of rare events and relies on a standard curve for an indirect measurement of the latent HIV reservoir.

To address these challenges, I planned to validate and optimize an automated PFGE platform that could eliminate unintegrated HIV fragments such as 2-LTR circles. I also wanted to use ddPCR to analyze HIV DNA from clinical samples to show that its absolutely measurement of the latent reservoir was accurate, precise, repeatable, and easy. Lastly, I was interested to see if the increased sensitivity of ddPCR could detect very low copy numbers in the latent HIV reservoir of a baby born to an infected mother. This case is particularly interesting because the baby was treated for a year but has tested negative since then. By performing these tests on well-studied and ongoing projects, my

ultimate goal was to better understand and further assess the pros and cons of our integrated HIV DNA assay.

MATERIALS & METHODS

Study Subjects

Samples of frozen PBMCs from chronically infected donors in the RAVEN project, who had been suppressed on ART for over a year, were stored in freeze medium containing 90% FBS and 10% Dimethyl Sulfoxide DMSO. Prior to DNA extraction, samples were rapidly thawed in a water bath at 37 °C.

Samples of frozen PBMCs from a four-year-old infant born to a chronically-infected HIV-positive mother was also stored and thawed as above. Additionally, CD4+ T lymphocytes were purified from the PBMCs using the EasySep Human CD4+ T Cell Isolation Kit from STEMCELL Technologies Inc. Cell count and phenotyping were performed with the Accuri C6 Plus from BD Biosciences.

HIV-1 Infected U937 (U1) cells obtained from the NIH AIDS Reagent Program have been used as a model to study HIV latency. The U1 cells contain an integrated proviral HIV genome that is defective in tat function as well as unintegrated 2-LTR circles. Following the NIH AIDS Reagent Program Guidelines, the U1 cells were propagated using 90% RPMI-1640 containing 2.0

mM L-Glutamine, 10% FBS, with Penicillin and Streptomycin added to a final concentration of 100 U/mL and 100 μg/mL respectively.

DNA Extraction

Cellular DNA was extracted using the QIAamp DNA Blood Midi Kit from QIAGEN. PBMCs or CD4+ T lymphocytes were washed and resuspended with PBS at 200 μL prior to the addition of the lysis buffer. Per the manufacturer's recommendation, two elution steps with Buffer AE were performed to maximize yield and the Buffer AE was allowed to incubate in the spin column for five minutes prior to centrifugation.

Ethanol Precipitation

1/10 volume of 3M Sodium Acetate, 2 μL of Glycogen, and 2 volumes of 100% Ethanol were added in order to the extracted DNA. Once the solution was gently mixed by inversion and stored at negative 20 °C overnight, the precipitate was centrifuged at >10,000 x g for 30 minutes at 4 °C and the supernatant was discarded. Following a wash with 1mL of freshly-made 70% Ethanol at >10,000 x g for 10 minutes at 4 °C, the supernatant was carefully removed without disrupting the DNA pellet and allowed to airdry. Finally, the dried DNA pellet

was resuspended with 35 μL of pre-heated Buffer AE at 60 °C from the QIAamp DNA Blood Midi Kit from QIAGEN.

Measuring DNA Concentration

1.5 μL of DNA was used on the NanoDrop One/OneC Microvolume UV-Vis Spectrophotometer from Thermo Scientific to estimate the DNA concentration (ng/ μL), the A260/A280 absorptivity ratio, and the A260/A230 absorptivity ratio.

BluePippen PFGE

The BluePippen platform from Sage Science was used to purify the high molecular weight DNA species. Before each run, the platform was cleaned with the manufacturer's wash cassette filled with DI water and optically calibrated with the provided fixture.

Two types of gel cassettes were tested to optimize the assay: the 0.75% Agarose, 4 – 20 kb High Pass Cassette and the High Pass Plus Cassette for >15 kb DNA Size Collections. The former allows a maximum DNA load of 5 ug diluted to a final volume of 30 μL (166 ng/ μL) and the latter allows a maximum DNA

load of 10 ug diluted to a final volume of 30 µL (332 ng/µL). The final run volume of 40 µL was obtained after the addition of 10 µL of loading solution.

For both gel cassettes, four lanes were utilized for the DNA samples and one lane was reserved for a molecular weight marker. 15 kb was set as the molecular weight cutoff mark and the total run time ranged from 4 to 6 hours depending on the gel cassette. Images of the two types of gel cassettes used and the program settings are shown in Figure 6 and Figure 7 below.

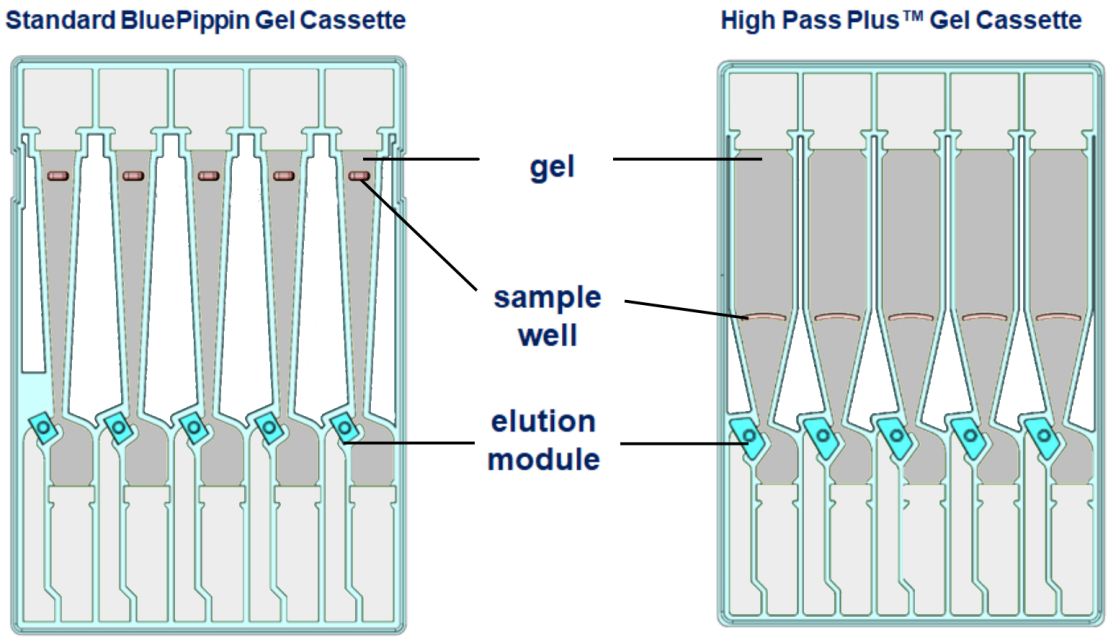

Figure 6. Diagram of BluePippen Gel Cassettes.

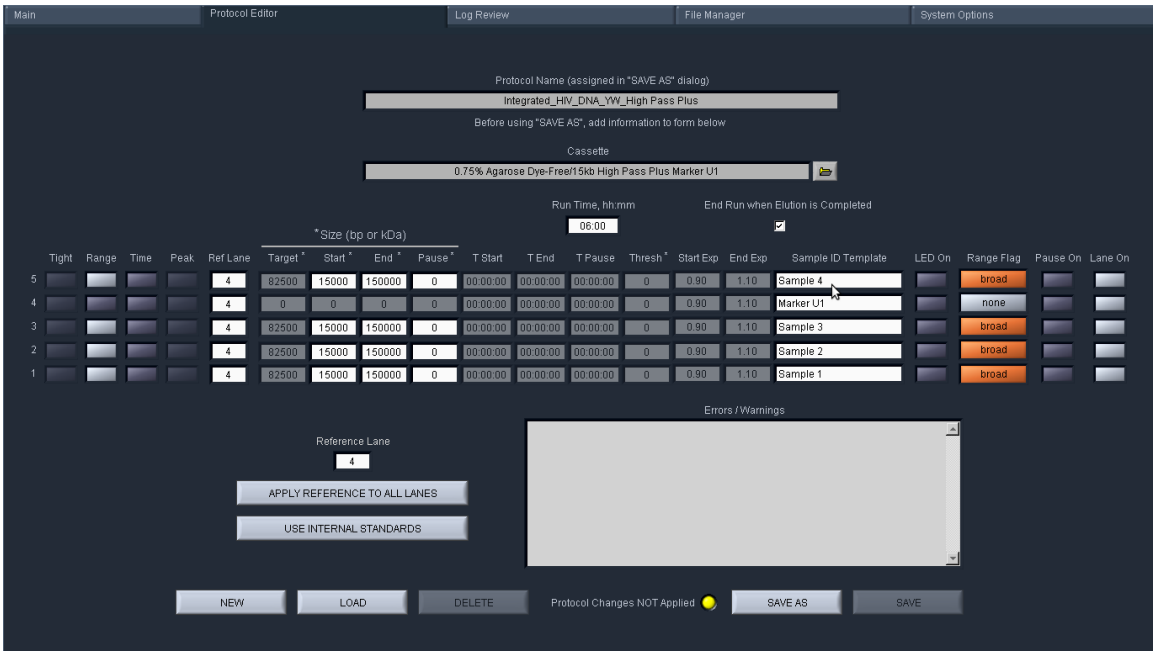

Figure 7. BluePippen High Pass Plus Gel Cassette Program Settings

Once the entire run was complete, the 0.75% Agarose High Pass Cassette yields a total of 40 µL of DNA eluate. 40 µL of 0.1% Tween 20 Buffer were used to rinse the remaining DNA in the elution well and added to the initial 40 µL for an end volume of 80 µL . The High Pass Plus Cassette, however, yields a total DNA eluate volume of 80 µL initially. Similarly, 80 µL of 0.1% Tween 20 Buffer were used as a rinse agent and combined into an end volume of 160 µL.

Concentrating Post-PFGE DNA

The purified DNA was then concentrated using the DNA 120 SpeedVac Concentrator from Thermo Scientific. The drying rate was set to 43 °C and the runs were repeated at 10-minute increments to make sure the DNA was not overly dried. In general, the desired volume was around 35 to 40 μL.

TapeStation Quality Control

To analyze the quality of our post-PFGE DNA samples, the concentration was normalized and loaded into the 2200 TapeStation system from Agilent. Using a Genomic DNA Screentape and DNA Reagents from the manufacturer, comparisons between the pre- and post-PFGE DNA samples were made and the analysis was visualized through the 2200 TapeStation Software.

Restriction Digest

The purified and concentrated DNA samples were restriction digested using the BanII enzyme in CutSmart Buffer from New England BioLabs. The thermocycler was programmed to incubate at 60 °C for 90 minutes before returning to a 4 °C infinite hold.

ddPCR

The following primer-probes were ordered from the Genomics and Sequencing Core at the Center of AIDS Research (CFAR) San Diego and used at an annealing temperature of 60 °C for the experiments.

Primer ID	Reporter & Quencher Dye	Sequence Name	Sequence (5' — 3')
Gag-HEX	HEX/ZEN/IBFO	1357 SK462 Gag-Forward	AGTTGGAGGACATCAAGCAGCCATGCAAAT
		1358 SK431 Gag-Reverse	TGCTATGTCAGTTCCCCTTGGTTCTCT
		1359 SK102 Gag-Probe	AGACCATCAATGAGGAAGCTGCAGAATGGGAT
2LTR-FAM	FAM/ZEN/BHQ	1217 MH535 2LTR-Forward	AACTAGGGAACCCACTGCTTAAG
		1218 MH535 2LTR-Reverse	TCCACAGATCAAGGATATCTTGTC
		1219 MH603 2LTR-Probe	ACACTACTTGAAGCACTCAAGGCAAGCTTT
Pol-FAM	FAM/ZEN/BHQ	1242 mf299 Pol-Forward	GCACTTTAAATTTTCCCATTAGTCCTA
		1243 mf302 Pol-Reverse	CAAATTTCTACTAATGCTTTTATTTTTTC
		1244 mf348 Pol-Probe	AAGCCAGGAATGGATGGCC
RPP30-HEX	HEX/ZEN/IBFO	RPP30-Forward	GATTTGGACCTGCGAGCG
		RPP30-Reverse	GCGGCTGTCTCCACAAGT
		RPP30-Probe	CTGACCTGAAGGCTCT

Table 2. Primer-Probe Information.

Digested high-molecular-weight DNA samples were emulsified into droplets by the QX200 ddPCR System from Bio-Rad following the manufacturer's protocol. 1000 ng of each sample was loaded in triplicate into a 96-well plate and sealed with a heated PX1 PCR Plate Sealer from Bio-Rad. Note that Low Retention Pippette Tips were also used from Mettler Toledo during sample loading to minimize adhesion.

Gag-HEX & 2-LTR-FAM *or* Gag-HEX & Pol-FAM Duplex			RPP30-HEX Singleplex		
Sample 1	Sample 9	Sample 17	Sample 1	Sample 9	Sample 17
Sample 2	Sample 10	Sample 18	Sample 2	Sample 10	Sample 18
Sample 3	Sample 11	Sample 19	Sample 3	Sample 11	Sample 19
Sample 4	Sample 12	Sample 20	Sample 4	Sample 12	Sample 20
Sample 5	Sample 13	Sample 21	Sample 5	Sample 13	Sample 21
Sample 6	Sample 14	Sample 22	Sample 6	Sample 14	Sample 22
Sample 7	Sample 15	Sample 23	Sample 7	Sample 15	Sample 23
Sample 8	Sample 16	Sample 24	Sample 8	Sample 16	Sample 24

Table 3. ddPCR Plate Map.

Each 96-well plate can hold a maximum of 24 samples; ¾ of the plate is used for the gag & 2-LTR or gag & pol duplex assay and ¼ of the plate is used for the RPP-30 singleplex assay. Taking into consideration the dynamic range of the QX200 ddPCR system, the digested DNA used for the RPP30 measurements was diluted 1:10 before amplification.

Immediately after the thermocycling steps (95 °C for 10 minutes, 40 rounds of 94 °C for 30 seconds followed by 58 °C for 60 seconds, and a final 98 °C for 10 minutes before returning to a 4 °C infinite hold), the ddPCR plate with emulsified samples was read with the QuantaSoft Software from Bio-Rad to prevent droplet degradation.

Data & Statistical Analysis

Raw fluorescence data were exported into the QuantaSoft Software from Bio-Rad for subsequent analysis. Thresholds were automatically or manually generated to distinguish positive, negative, or ambiguous signals on a 1- or 2-D graph following a custom algorithm. False positives visualized from a negative control and extreme outliers were excluded from the final data set. ddPCR template copies per sample were calculated by averaging all replicate wells of a particular sample. The number of RPP30 template copies per sample was used to compute the total number of cells per sample.

Statistical analysis was done using the GraphPad Prism 8.0 Software. HIV DNA detection by ddPCR before and after PFGE was compared with Wilcoxon's paired nonparametric t-test.

RESULTS

PFGE Purification

DNA with a size of >15 kb from U1 cells was purified with PFGE and characterized before and after with the 2200 TapeStation. Figure 8 illustrates the functional removal of 2-LTR circles from the HIV provirus in U1 cells. Across multiple replicates of both the 0.75% Agarose Gel Cassette and the High Pass Plus Cassette, a significant amount of DNA <15 kb was filtered out into the electrophoresis buffer flow-through.

Size Exclusion Gel Cassettes

Using all five lanes, the 0.75% Agarose High Pass Cassette ran for about six hours on average. In contrast, the High Pass Plus Cassette only required about four hours.

Both gel cassettes resulted in a substantial decrease of <15 kb DNA species following the manufacturer's default protocols; however, NanoDrop One/OneC measurements in Table 4 illustrate that the resultant yield for the former gel cassette has an average recovery of around 40% whereas the latter gel cassette has an average recovery of around 70%.

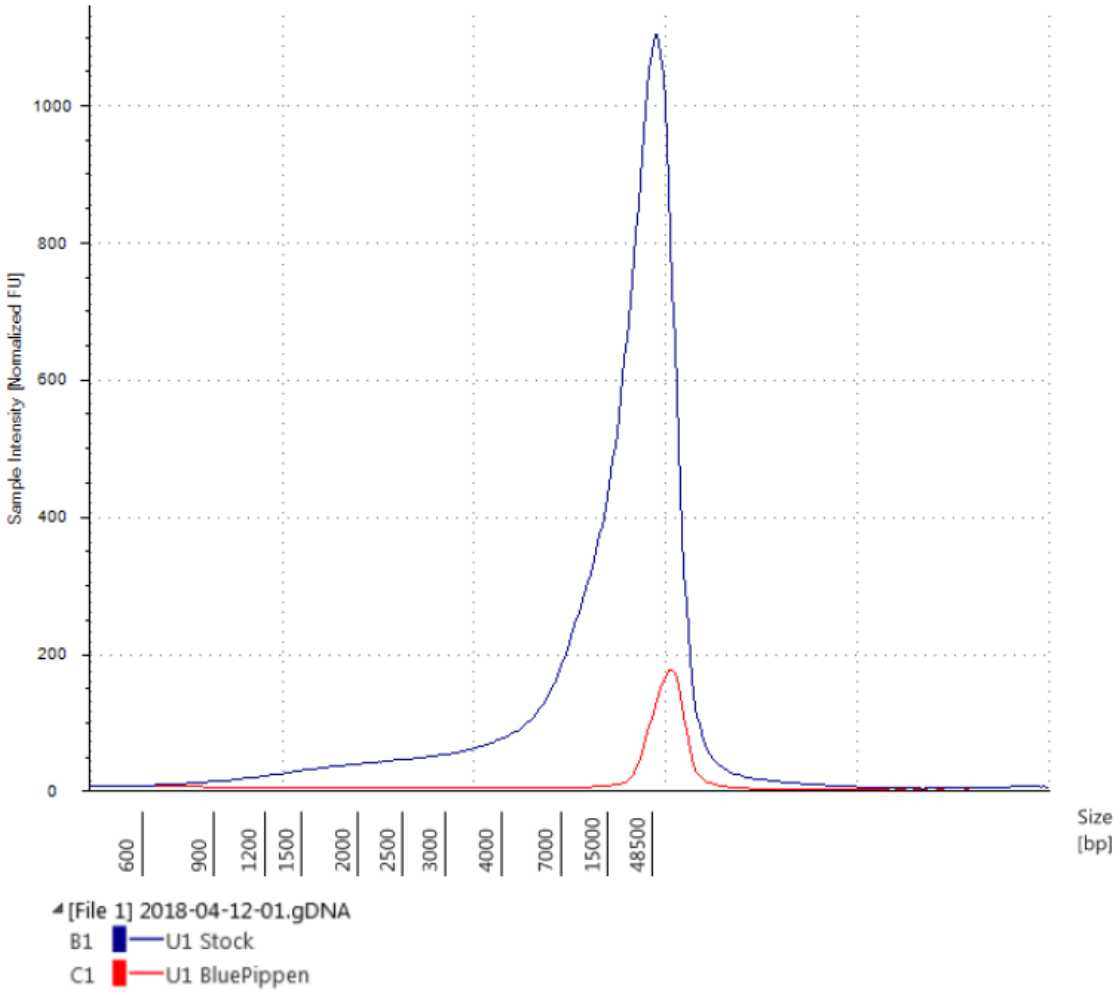

Figure 8. PFGE Electrophoregram.

Comparison of DNA from U1 cells before and after running PFGE with the 0.75% Agarose High Pass Cassette. Peaks for the U1 Stock and U1 BluePippen were measured at 51.468 kb and >60 kb, respectively. The electrophoregram was generated by the 2200 TapeStation Software and DNA concentrations were not normalized in this figure.

Gel Cassette	Input DNA (ng)	Output DNA (ng)	Yield (%)	Average Yield (%)
0.75% Agarose High Pass	4101	1896	46	42
	4101	1192	29	
	2895	1752	61	
	2895	960	33	
High Pass Plus	6292	5009	80	71
	6292	4227	67	
	9622	6784	71	
	9622	6327	66	

Table 4. DNA Recovery Post-PFGE.

HIV DNA Detection by ddPCR Between Gel Cassettes

DNA was extracted from 4 PBMC samples from infected donors undergoing ART with suppressed viral loads. HIV gag copies were measured and calculated before and after PFGE using the 0.75% Agarose High Pass Cassette and the High Pass Plus Cassette. Comparing the data for the gel cassettes, Figure 9 illustrates that the 0.75% Agarose High Pass Cassette detects more HIV gag copies per 1 million cells. However, the difference is not significant ($P = 0.25$).

HIV DNA Detection by ddPCR Before and After PFGE

Despite the removal of lower molecular weight HIV DNA which contain gag copies and the further shearing of higher molecular weight HIV DNA by the

BluePippen PFGE platform, Figure 9 still illustrates that the calculated gag copies per 1 million cells for all samples increased after purification by PFGE. Still, the increase from both gel cassettes was not significant (P = 0.125 for both the 0.75% Agarose High Pass Cassette and the High Pass Plus Cassette).

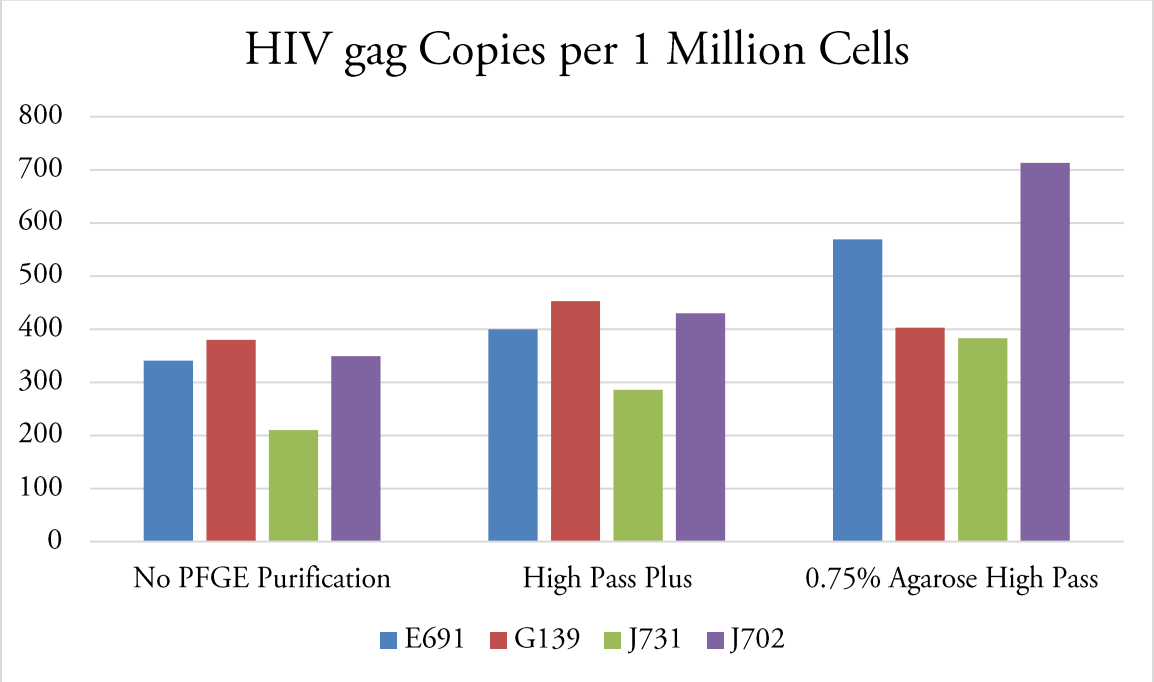

Figure 9. Comparison of HIV gag Copies per 1 Million Cells by ddPCR

ddPCR's Improved Sensitivity for Rare Event Detection

We wanted to assess the sensitivity of our assay with these samples since the detection of extremely low copy numbers of HIV DNA is challenging. PBMCs from a child born to an infected mother were tested for the latent HIV reservoir using PFGE and ddPCR. In clinic, the child's viral load was undetectable and its CD4+ T lymphocyte count was normal. We were interested to study this subject because the Mississippi Baby had been thought to be successfully cured before HIV DNA was detected again in her blood. ddPCR data from a previous technician on PBMCs prior to 07/23/2019 detected low positive signals for HIV DNA copies of pol and gag. However, there was no consistent trend and the highest number of detected HIV DNA copies was calculated to be in the double digits.

For the newest batch of PBMCs, CD4+ T lymphocytes were isolated to increase the sensitivity and accuracy of the assay; moreover, PFGE was utilized to remove unintegrated HIV species from the extracted DNA. By using an adequate amount (1000 ng) of DNA material per well and increasing the number of replicates per sample, Table 5 illustrates that ddPCR can achieve a limit of detection of <1.

Sample	Limit of Detection	pol Copies per 1 Million Cells	gag Copies per 1 Million Cells
Positive Control (07/23/2019)	2.396357537	997.71	829.14
Negative Control (07/23/2019)	2.798768542	0	0
CD4+ T Lymphcytes (07/23/2019)	0.999263749	0	0
PBMCs (05/20/2019)	3.753753754	42	31
PBMCs (11/08/2018)	0.354609929	0.77	1.28
PBMCs (02/18/2017)	1.284026708	3.08	6.16
PBMCs (05/19/2016)	5.341880342	0	0

Table 5. Summary of ddPCR Results from Child.

Reproducibility and Robustness of ddPCR from RAVEN Samples

PBMCs from two HIV-positive donors and one HIV-negative donor were tested for copies of gag by ddPCR. Figure 10 illustrates three complete technical replicates that were run with the same samples under identical conditions. Figure 8 illustrates no statistically significant difference between runs (P = 0.25). The standard deviations of the Negative Control, RAVEN 3, CARE 215 were 0, 31.04, and 198.3 respectively.

To test the robustness of ddPCR and its ability to measure changes in the reservoir, a limiting dilution was done with the RAVEN 3 sample in 12 replicates. 500000 CD4+ T lymphocytes were plated in the first row and 1:3 dilutions were performed until the last row. Figure 11 illustrates that changes in the size of the latent HIV reservoir can be measured.

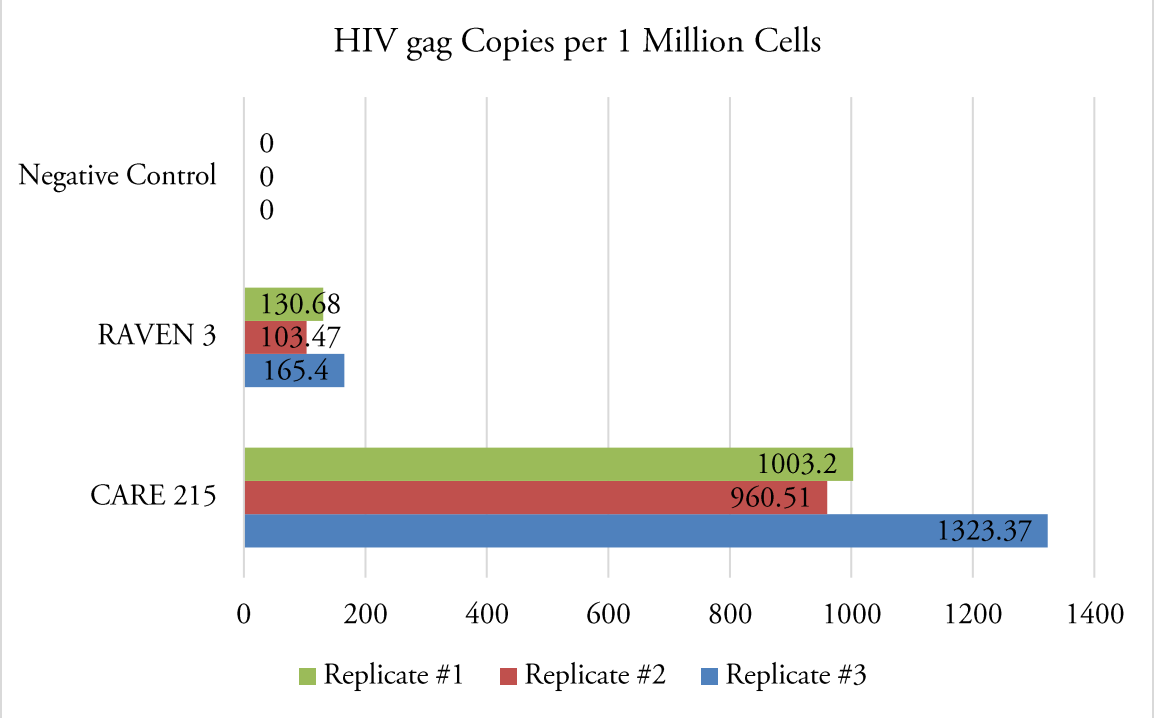

Figure 10. Reproducibility of ddPCR Measurements across Three Replicates.

Dilution	CD4s per Well	RAVEN 3 HIV gag Copies per 1 Million Cells			
1	500000	2072	2035	2849	3971
2	166667	1344	2578	2380	1406
3	55556	384	495	165	516
4	18519	19	0	221	20
5	6173	0	7	0	0
6	2058	0	14	0	7
7	686	6	7	0	0
8	229	0	0	0	0
		Replicate #1	Replicate #2	Replicate #3	Replicate #4

Dilution	CD4s per Well	RAVEN 3 HIV gag Copies per 1 Million Cells			
1	500000	1566	1912	2097	2060
2	166667	1418	2528	2602	999
3	55556	310	397	777	146
4	18519	52	100	93	70
5	6173	7	20	6	6
6	2058	0	7	0	0
7	686	0	0	0	0
8	229	0	0	0	0
		Replicate #5	Replicate #6	Replicate #7	Replicate #8

Dilution	CD4s per Well	RAVEN 3 HIV gag Copies per 1 Million Cells			
1	500000	3342	3601	5451	8300
2	166667	3379	3071	1073	1640
3	55556	456	1233	395	530
4	18519	12	11	23	63
5	6173	65	0	0	6
6	2058	0	0	0	0
7	686	0	11	0	0
8	229	0	0	6	0
		Replicate #9	Replicate #10	Replicate #11	Replicate #12

Table 6. ddPCR Detection of Changes in HIV DNA by Limiting Dilution.

DISCUSSION

PFGE and Integrated HIV DNA

By using the automated BluePippen PFGE platform, we removed a large fraction of lower molecular weight DNA species from our samples. The 15 kb cutoff causes episomal HIV DNA such as 1- and 2-LTR circles to be almost completely eliminated from subsequent ddPCR analysis, and should therefore lead to a more accurate measurement of integrated DNA in the latent HIV reservoir. TapeStation 2200 electropherograms also show a similar purification effectiveness between DNA extracted from cell lines and DNA extracted from patient samples.

Compared to the Alu-gag qPCR assay, findings from previous laboratory colleagues have shown that results correlate well and are comparable (Lada et al., 2018). Unlike traditional direct current (DC) agarose gel electrophoresis, the BluePippen PFGE platform utilizes alternating current (AC) that allow for changes in current direction based on specific time intervals. Theoretically, this allows for an improved separation between the different molecular weight DNA species. In addition, the pre-made gel cassettes separate each lane with a plastic blockade and prevents contamination between precious samples. Finally, this

automated platform allows for the user to complete other experiments during the PFGE run. The program will automatically stop once elution is complete and samples can remain in the well safely for hours with minimal evaporation

Improving Upon the 0.75% Agarose High Pass Gel Cassette

In the original designed protocol, the 0.75% Agarose High Pass Gel Cassette was used. Normally, ddPCR assays recommend a load of 1000 ng per well to maximize sensitivity to increase the limit of detection. However, only 5 ug of genomic DNA maximum could be loaded into each lane per the manufacturer's recommendations. This means that an input of 5 ug of DNA would typically lead to an output of 2 ug of high molecular weight DNA, sufficient only for two ddPCR replicates. Furthermore, the median recovery rate of around 40% further complicates this issue. Keeping in mind that one lane per gel cassette is required for the molecular weight marker and that each run required about 6 hours to complete, this process would clearly be time-consuming and expensive for a large cohort of samples or if a high amount of DNA is required.

With the RAVEN project totaling around 120 PBMC specimens, we decided to test the new High Pass Plus Gel Cassette from BluePippen to optimize

our workflow. Though the gel cassette can still process a maximum of four samples per run, the initial load could now be doubled to 10 ug of DNA. Even if the recovery remained at 40%, we would have enough DNA post-PFGE to run our ddPCR samples in triplicates. However, experiments with both U1 DNA and genomic DNA have shown that the High Pass Plus Gel Cassette has an DNA recovery of closer to 70%, allowing us to have more DNA to work with after the removal of episomal DNA species such as 1- and 2-LTR circles.

The run time on the High Pass Plus Gel Cassette has also been reduced compared to its counterpart. Most runs were complete near the three-and-a-half-hour mark, with some pushing the four-hour mark. For laboratories with just one BluePippen machine, up to eight samples could still be easily processed within a day. Although it has been suggested that a second run can be completed overnight on the 0.75% Agarose High Pass Gel Cassette, the manufacturer specifically noted that gel cassettes should not be left in the machine overnight. This is because the evaporation of the electrophoresis buffer could cause damage to the LED electrodes responsible for size separation and that the quality of the eluted DNA product could be compromised if not suspended in a proper amount of buffer.

Quality of Integrated DNA from the High Pass Plus Gel Cassette

Initially, we had worries that a high DNA load with a shorter run time would result in insufficient removal of low molecular weight DNA species. However, comparing both U1 DNA samples as well as genomic DNA samples from chronically-infected HIV patients, the High Pass Plus Gel Cassette was an equally effective option for DNA size selection.

It is important to note that one downside of the High Pass Plus Gel Cassette is that the cutoff value can only be set above 15 kb. Laboratories with a specific need to keep DNA species <15 kb should use the 0.75% Agarose High Pass Gel Cassette, since it is much more flexible in its start and stop separation points. Users of the BluePippen platform should also remember to update their machine's firmware as the pre-programmed protocol for the High Pass Plus Gel Cassette is available only in the latest version.

Using ddPCR to Maximize Sensitivity and Reproducibility

As mentioned before, qPCR has been the most common PCR assay for measuring the latent HIV reservoir. However, its quantitation methods are indirect. Depending on experimental design and conditions, only 20% to 70% of the total integration events can be detected in a given alu-gag qPCR run. To

correct for this, a correction factor generated from a standard integration cell line is multiplied onto the original results (Liszewski et al., 2009).

PFGE with ddPCR offers a potential solution for this issue. It allows for a direct measurement of DNA copies within each well without the need for post-analysis statistical correction. Data published from multiple studies have also noted the increased sensitivity, reproducibility, precision, accuracy, and scability compared to qPCR assays (Strain et al., 2013; Zhao et al., 2016) .

In our collaboration with UTHealth to measure the latent HIV reservoir for the infant born to an infected mother, it was crucial that we used ddPCR for our measurements. We needed to maximize sensitivity as the HIV copy frequencies for gag and pol were at most in the single digits. The results did initially seem to suggest that the latent HIV reservoir of the infant had risen since 2016; but having tested the most recent PBMC and CD4+ T lymphocyte samples in nine replicates, we are more confident that the previous data may have been a result of contamination or technical erros.

For a larger cohort of samples like the RAVEN project, we were interested to confirm that ddPCR could offer unparalleled reproducibility Not only did our results show an extremely low variance between each sample per run, even the

variance between replicate wells has been shown to be relatively small with properly-set thresholds and the removal of false positives.

Figure 12 illustrates a composite 2D scatter plot of 24 ddPCR wells with a manually set threshold. The X-axis illustrates positive events for HIV gag copies (HEX dye) and the Y-axis illustrates positive events for HIV tat/rev copies (FAM dye). False double-positive events like the single one below can be removed prior to subsequent calculation of HIV DNA copies per 1 million cells for improved accuracy.

Figure 11. QuantaSoft 2D Scatter Plot

Limitations and Future Applications of PFGE

The implementation of the High Pass Plus Gel Cassette has definitely helped us to optimize our workflow. However, although we can purify high molecular weight DNA species with an improved recovery in less time, there are still many checkpoints that must be done to ensure that the PFGE runs smoothly. Perhaps the most crucial is the prevention of air bubbles in the pre-made gel cassette that can completely ruin separation and elution in a lane. We have yet to encounter this issue in our experiments, but users should carefully inspect for air bubbles behind the elution wells and remove them before proceeding. Other important checkpoints include thoroughly cleaning the PFGE platform with a rinse cassette and calibrating the LED electrodes before each run.

One potentially unexplored use of the BluePippen PFGE platform by is its ability to collect the low molecular weight DNA flow-through in a separate chamber. The eluates in this chamber include 1- and 2-LTR circles as well as high molecular weight DNA that has been sheared by the PFGE process. If one desires to analyze and measure unintegrated DNA in addition to integrated DNA, these gel cassettes could certainly prove useful. The low molecular weight DNA species are highly diluted in electrophoresis buffer but can be concentrated by ethanol precipitation or with a vacuum concentrator. Downstream ddPCR

analysis can also be carried out for the low molecular weight DNA species to verify the purification of 1- and 2-LTR circles in the flow-through.

Limitations and Future Applications of ddPCR

ddPCR results generated in this project comes exclusively from the Bio-Rad ddPCR platform. Despite the the high cost of instruments and reagents, the QX200 does still detect false positive events during almost every reading that skews the accuracy of the data. Even in non-template negative control wells, false positive signals can be seen frequently. Moreover, the default thresholds generated by the Bio-Rad QuantaSoft Software also lead to incorrect calls in categorizing an event as positive or negative. However, the majority of these errors can be addressed by manually re-analyzing the raw fluorescence data. Still, we are not certain if these errors are specific to the Bio-Rad ddPCR platform or due to some shared aspects of the ddPCR process.

That said, ddPCR is still a robust method of measuring integrated HIV DNA compared to the Alu-gag qPCR. The RAVEN project, upon completion, will allow for the statistically comparison of data between multiple laboratories to confirm the intra-lab precision and inter-lab reproducibility of ddPCR for the quantification of the latent HIV reservoir. With the multitude of HIV cure

strategies still being developed and tested, I firmly believe that ddPCR paired with PFGE will become be an attractive option to detect changes within the latent HIV reservoir.

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